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IN VITRO DIRECT REGENERATION FROM COTYLEDONARY NODE OF *SAUSSUREA LAPPA* CLARKE – A VALUABLE ENDANGERED MEDICINAL HERB

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ABSTRACT

Saussurea lappa is a pharmacologically important endangered plant enlisted in CITES Appendix I as well as in NMPB as important species of high trade and stable cultivation. An efficient protocol for *in vitro* multiplication of *Saussurea lappa* was developed from cotyledonary node explants. Multiple shoots were induced from cotyledonary nodes excised from *in vitro* raised seedlings on Murashige & Skoog's medium (MS medium) containing either BAP (6-benzylaminopurine) or Kn (Kinetin) in alone or in combination with NAA (1-Naphthaleneacetic acid). High-frequency average bud break (83.33%) and maximum number of shoots per explant (5.9 shoots) were recorded with medium supplemented with BAP + NAA at an optimum concentration of 2 mg/l + 0.1 mg/l respectively. For successful rooting, regenerated shoots were transferred to half strength and full strength MS medium containing various concentration of NAA. Best root formation (85%) was achieved with half strength MS medium supplemented with 0.5 mg/l NAA. The rooted plants were transferred in plastic cups containing vermiculite: sand (3:1) and were maintained in high humidity. Eighty per cent of the plantlets survived in natural conditions.

KEYWORDS: Micropropagation, Cotyledonary Node, *In Vitro*, *Saussurea lappa*

INTRODUCTION

Saussurea lappa Clarke (commonly known as “Kuth” or “Costus”) belongs to family Asteraceae is an endangered medicinal herb enlisted in CITES Appendix – I (The Convention on International Trade in Endangered Species) as well as in NMPB (National Medicinal Plants Board, Govt. of India) as important species of high trade and stable cultivation. It is a divine therapeutic perennial plant reported from the high altitude regions of Jammu & Kashmir and Himachal Pradesh (Aswal & Mehrotra, 1994). The dried root extract is utilized in the formulation of many medicines to counter the number of aliment. It is a well known for anti-microbial, anti-ulcer, anti-inflammatory, anti-cancer and anti-hepatotoxic properties (Madhuri *et al*., 2012). It is effective against common cold, cough, fever, asthma, diarrhea, skin diseases, heart diseases and nervous disorders (Zahara *et al.*, 2014).

Owing to its far-ranging use, plant was over uprooted from wild resulting it to the eve of extinction. This plant mainly reproduces by seeds and roots. Moreover, we too reported the high mortality of seedlings in early stages added a constraint in conventional propagation through seeds when raised in field conditions. The propagation by root cutting has too its limitations, because they are mainly exploited for medicinal purpose. Thus, micropropagation is an efficient method for mass production of *Saussurea lappa*.

MATERIALS AND METHODS

The seeds were procured from Lahaul valley of Himachal Pradesh (India). Healthy seeds were sorted out and washed under running water to remove the adhering dust. The seeds were then treated with liquid detergent (Tween-20) and surface sterilized with 0.1% (w/v) freshly prepared mercuric chloride solution for seven minutes under sterile environment. Then, seeds were washed with four times with sterilized double distilled water. Surface sterilized seeds were inoculated on half strength MS medium (Murashige & Skoog, 1962) containing 3% sucrose, 0.8% agar and pH adjusted to 5.8. The cultures were incubated at 25±2 ^{o} C with 55–65% relative humidity under a 16-h light/8-h dark cycle at 27 μM $m^{-2}s^{-1}$ PAR light intensity from 6500 K colour temperature white fluorescent tubes.

Cotyledonary nodes excised from two week old seedlings were used as explants (Figure -1 A). The explants were cultured on MS medium supplemented with 3% sucrose, 0.8% agar containing various concentrations of the growth regulators such as BAP (6-benzylaminopurine), Kn (Kinetin) and NAA (1-Naphthaleneacetic acid) in alone or in combination. The pH of the medium was adjusted 5.8 before autoclaving and cultures were maintained under similar conditions of seeds germination. The primary shoot buds obtained from each harvest at an interval of 40 days were cut into single buds and transferred to fresh MS medium for axillary shoot proliferation.

The proliferated shoots apex with leaves were excised and cultured for root regeneration on half strength and full strength MS medium supplemented with 3% sucrose, 0.8% agar and various concentrations of NAA. The well rooted plantlets were gently washed with soft brush under running tap water to remove the adhering agar with minimum injury. Plantlets were then transferred to small plastic cups containing sterile vermiculite: sand (3:1) potting mixture. The plantlets were covered with a glass jars to maintain high humidity around the plants. They were supplied with half strength MS salt solution on alternate days. In third week, glass jars were removed for 3-4 hrs daily to expose the plants to the field conditions. After about 4 weeks these plants were transferred to bigger pots and were maintained under natural field conditions of photoperiod and temperature.

All the experiment was conducted with a minimum of twenty replicates per treatment and was repeated three times. The data were statistically analyzed using (SPSS) one-way analysis of variance (ANOVA) and the differences contrasted using a Duncan's multiple range test at $P \leq 0.05$.

RESULTS

The cotyledonary nodes in MS medium without growth regulators (Control) do not result into any significant morphogenetic response. The addition of plant growth regulators showed the positive response in term of per cent bud break, number of days required for bud break and number of shoots per explant (Table -1). The medium supplemented with BAP (0.5, 1.0, 1.5, 2.0 mg/l) and Kn (0.5, 1.0, 1.5, 2.0 mg/l) alone showed increase in regenerative response with the increase in the concentration of growth regulators. The maximum response among all the mediums supplemented with BAP and Kn alone, reported higher regeneration proliferation at higher concentrations. The medium supplemented 2.0 mg/l BAP showed about 78.33% bud break in average 24.45 days by producing about five shoots per explant where as medium supplemented with 2 mg/l Kn produced 60% bud break in average 26.90 days by producing about 2.90 shoots per explant (Table – 1). The medium supplemented with combination of BAP + NAA also showed comparatively better response than medium combinations of Kn + NAA. Among BAP and Kn, BAP proved to be more efficient in all the factors tested.

The best result for multiple shoot production was observed in medium containing BAP + NAA (2.0 mg/l + 0.1 mg/l) with highest recorded average bud break (83.33%), less number of days required for bud production (24.25) and highest number of shoots per explant (5.90).

For root induction, *in vitro* raised shoots protuberance with leaves (0.5 - 1 cm) were excised and cultured on full and half strength MS medium supplemented with or without various concentrations (0.5 mg and 1.0 mg/l) of NAA (Table 2). MS medium (Full and Half) devoid of NAA does not showed any significant result. The higher concentrations of NAA and MS salts favored the callus formation. The best result in term of average per cent rooting (85%) and number of healthy long thin roots formed (6.4) was observed in half strength MS medium supplemented with 0.5 mg/l NAA (Figure – 1 C). After about 40 days of culture in rooting medium, plants were gently removed out from rooting medium and washed carefully with sterilized water using a soft brush to remove the adhering agar-agar. These plants were acclimatized and hardened (Figure -1 D, E, F). Eighty per cent of the plants survived well in natural conditions.

Table 1: Effect of Various Concentrations of Plant Growth Regulators Alone or in Combination in MS medium on Shoot Initiation from Explants.

Growth Regulator (mg/l)	Bud Break (%)	No. of Days Required for Bud Break	No. of Shoots
Control	10.00^{j}	30.70^{f}	1.00^{d}
BAP (0.5)	51.66^{f}	25.75^{abcd}	2.78^{cd}
BAP (1.0)	61.66^{de}	25.25^{abc}	3.50^{ab}
BAP (1.5)	71.66^{c}	24.90^{ab}	4.70^{bcd}
BAP (2.0)	78.33^{b}	24.45^{a}	5.00^{a}
Kn (0.5)	26.66^{i}	27.95^{e}	1.15^{d}
Kn (1.0)	35.33^{h}	27.50^{de}	1.20^{d}
Kn (1.5)	46.66^{g}	27.25^{cde}	1.35^{d}
Kn (2.0)	60.00^{e}	26.90^{bcde}	2.90^{cd}
BAP (2.0) + NAA (0.5)	66.66^{cd}	25.20^{abc}	3.45^{bc}
BAP (2.0) + NAA (0.1)	83.33^{a}	24.25^{a}	5.90^{a}
Kn (2.0) + NAA (0.5)	56.66^{e}	26.05^{abcde}	2.05^{de}
Kn (2.0) + NAA (0.1)	66.66^{cd}	24.95^{ab}	3.00^{cd}

Mean values followed by different letters within a column don't differ significantly at $P \leq 0.05$ according to Duncan's Multiple Range Test

Table 2: Effect of Half Strength MS Medium and Full Strength MS Medium With or Without Various Concentrations of NAA in Root Induction

Medium	Plant Growth Regulator	Rooting (%)	No. of Roots	Root Morphology
MS half strength	-	-	-	-
MS half strength	0.5 mg/l NAA	85^{a}	6.4^{a}	Long, thin and very less callus formation
MS half strength	1.0 mg/l NAA	60^{b}	4.5^{b}	Short, thick and less callus
MS full strength	-	-	-	-
MS full strength	0.5 mg/l NAA	50^{c}	3.9^{c}	Very short, thick and high callus formation
MS full strength	1.0 mg/l NAA	35^{d}	2.8^{d}	No rooting and Profuse callus formation

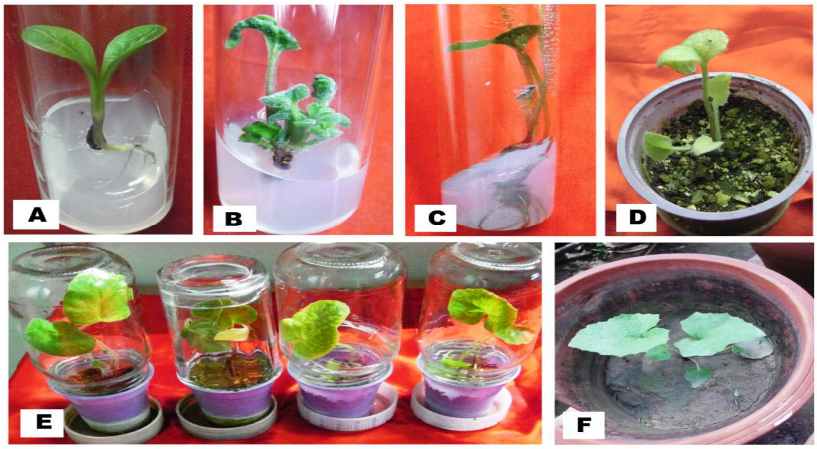

Figure 1: A *In Vitro* Raised Seedling, B – Multiple Shoots Emerging on Cotyledonary Nodal Segment on Medium Supplemented with BAP + NAA (2 mg/l + 0.1 mg/l), C – Rooting on Excised Shoot on Medium Supplemented with 0.5 mg/l NAA, D – Transferred Plantlet in Vermiculite: Sand (3:1), E – Plantlets Covered with Glass Jars to Maintain High Humidity & F – Plants Transferred to Bigger Pots in Field Condition

DISCUSSIONS

Plant tissue culture is a fast alternative to conventional multiplication methods and is used as a complementary strategy for conservation and utilization of genetic resources (Groach *et al*., 2014a). Cotyledonary nodes have generally proved to be an excellent starting material in plant species to induce multiple shoots through axillary branching (Meena *et al*., 2014). In our case, experimental analysis indicated that among different combinations tested, the effective shoot multiplication from cotyledonary explants of *S. lappa* was found in MS medium fortified with 2.0 mg/l BAP + 0.1 mg/l NAA (Fig. 1B). However, another better result was observed in MS medium supplemented with BAP alone at optimum concentration of 2.0 mg/l. Kn in alone and in combination proved to be less effective than BAP. This proves the supremacy of BAP effectiveness over Kn in shoot proliferation. The similar findings of BAP effectiveness alone or in combination with lower concentration of auxin is one of the most successful and widely used protocols for shoot regeneration in numerous reports i.e. Koroch *et al.*, (2002) in *Echinacea purpurea*, Alam *et al.*, (2010) in *Ricinus communis*, Bermejo *et al.*, (2012) in *Lens culinaris*, Tang *et al*., (2012) in *Vigna unguiculata*, Higuchi & Amaki (1989) in *Asplenium nidus*, Groach *et al.*, (2014b) in *Vitex negundo*, Nabi & Shrivastava, (2015) in *Psoralea corylifolia*.

The rooting is a necessary step after shoot regenerations to complete the all organs of plant to sustain in field conditions. NAA is widely used as rooting inductor in *in vitro* cultures. In present study, medium devoid of growth regulator does not resulted into any significant rooting. Among two different concentration of MS medium (Half and Full strength) with NAA, Half strength MS medium supplemented with 0.5 mg/l of NAA proved to be best for rooting. This is consistent with the study of Singh *et al.*, (2013) in *Punica granatum.* They observed maximum root formation with half strength MS medium supplemented with 0.5 mg/l NAA + 200 mg/l activated charcoal as compared with the full strength MS medium with same concentration of NAA. Andrade *et al.*, (1999) reported rooting rates and root growth increased with increased concentrations of NAA and the reduction of the salt strength of the medium. The similar reports of use of NAA as successful rooting has been reported in George & Rao, (1980) in *Brassica juncea*, Bahrany (2002), in *Citrus aurantifolia*, Chakravarty & Goswami, (1999) in *Citrus acida*, Behera & Sahoo, (2009) in *Saccharum officinarum.*

CONCLUSIONS

This experimental study has resulted in an expeditious and reproducible regeneration protocol of *Saussurea lappa* from cotyledonary nodal explants. It involves raising of aseptic cultures on MS medium and multiple shoot proliferation on MS medium supplemented with 2 mg/l BAP + 0.1 mg/l NAA and subsequent transfer to best rooting on half strength MS medium fortified with 0.5 mg/l NAA. The plants were acclimatized to natural field conditions. To our knowledge, this is a best report of multiple plant shoot regeneration from cotyledonary nodes which can prove helpful in the establishment of a fast alternative regeneration system to conventional propagation methods and further basic as well as applied biotechnological studies.

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